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DNA Is Packaged within Membrane-Derived Vesicles of Gram-Negative but Not Gram-Positive Bacteria

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Recently, DNA packaged within nuclease-resistant membrane vesicles of *Neisseria gonorrhoeae* and *Borrelia burgdorferi* was described. This study assayed 18 species of gram-negative and gram-positive eubacteria for nuclease-protected DNA associated with extracellular membrane vesicles. Vesicles from only the gram-negative bacteria contained nuclease-protected linear or supercoiled DNAs or both.

The formation of membrane vesicles is common among bacteria, and the characteristics and several possible functions of extracellular vesicles have been reviewed in detail elsewhere (11). Recently, the export of DNA associated with extracellular membrane vesicles was demonstrated in *Neisseria gonorrhoeae* and *Borrelia burgdorferi* (4, 7). In both species, the DNA was protected from exhaustive treatment with pancreatic DNase I, and it appeared to be enclosed within the vesicles. Furthermore, when wild-type recipient gonococci were incubated with DNase I and vesicles purified from penicillinase-producing *N. gonorrhoeae*, R plasmids were efficiently incorporated and expressed by the recipients, suggesting that such vesicle-mediated transfer of DNA constitutes a previously undocumented genetic exchange mechanism (4).

Subsequent experiments identified DNA-binding proteins specifically expressed in two unique fractions of gonococcal vesicles, designated BI and BII (3). Outer-membrane-derived BII vesicles contain at least four DNA-binding proteins (3), and these vesicles protect enclosed plasmids from nuclease digestion (4). Fraction BI, derived from the cytoplasmic membrane, contains an 11-kilodalton protein that may contribute to binding and uptake of transforming DNA (3). Such findings with fraction BI appear to correlate with detailed studies of *Haemophilus* transformasomes. Transformasomes are membrane evaginations on the surface of competent *Haemophilus influenzae* cells that mediate DNA uptake (2, 9). Our observations suggest that the export of DNA within outer-membrane-derived vesicles and vesicle-mediated genetic exchange are distinct from the uptake of external DNA by transformasome structures, since the exchange of vesicle-associated plasmids by *N. gonorrhoeae* was independent of transformation by exogenous chromosomal DNA (4). Possible correlations between (i) vesicle-mediated exchange and the presence of a conjugative plasmid in donor gonococci and (ii) the relatively high efficiency of plasmid transfer suggest that interactions between DNA and extracellular bacterial membrane vesicles may be genetically regulated and biologically significant (3, 4).

In order to determine whether the export of membrane-vesicle-associated DNA is characteristic of eubacteria, we surveyed 15 genera and 18 species of gram-negative and gram-positive bacteria for the presence of extracellular vesicles and for packaged DNA. Log-phase agar cultures were examined by electron microscopy, and vesicles were isolated from the species producing them. After exhaustive digestion with DNase I, any DNAs present in the vesicle preparations were purified and then characterized by electron microscopy and gel electrophoresis.

The bacterial species and strains used are listed in Table 1. Type or neotype strains were purchased from the American Type Culture Collection and maintained according to the recommendations of that organization. *H. influenzae* and *N. gonorrhoeae* were maintained on gonococcal clear typing medium (5) with or without 1 g of hemoglobin per liter of medium, respectively. *B. burgdorferi* was maintained in BSK II medium (1). For experiments, log-phase cultures were harvested, and cells and vesicles were separated by differential and sucrose density gradient centrifugation, as previously described (4, 7).

Prior to nucleic acid determinations, purified extracellular membrane vesicles were suspended in Dulbecco phosphate-buffered saline (pH 7.2) supplemented with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and were then incubated with 10 μg of pancreatic DNase I per ml (2.5 U/μg) (Worthington Diagnostics, Freehold, N.J.) for 10 min at 25°C (4, 7). Such treatment was previously shown to degrade extraneous DNA in vesicle preparations (4).

For electron microscopy, bacterial cells and vesicles were suspended in the supplemented Dulbecco phosphate-buffered saline, adsorbed to Parlodion (Mallinckrodt, Inc., St. Louis, Mo.)-coated grids, and negatively stained for 10 s with 0.5% ammonium molybdate at pH 6.5. Alternatively, colonies were blotted onto coated grids and then stained as described above. The grids were then examined at 75 kV. The Kleinschmidt mounting technique for nucleic acid electron microscopy, with adaptations described by Garon (6), was used to examine DNA extracted from vesicles and bands excised from agarose electrophoretic gels.

Cellular and vesicle-associated DNAs were purified from sodium dodecyl sulfate-proteinase K lysates by phenol-chloroform extractions (10). Any RNA present in the extracts was hydrolyzed with DNase-free RNase (Sigma Chemical Co., St. Louis, Mo.) (10). Agarose gel electrophoresis of extracted DNA was performed as previously described (4, 10).

Log-phase cultures of each organism considered were observed by electron microscopy for membrane vesicles associated with cell surfaces. Negative stains of cells and purified vesicles from *Bordetella pertussis* are shown in Fig. 1. Gram-negative bacteria such as *B. pertussis* (Fig. 1a) had vesicles both on cell surfaces and in surrounding areas.

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TABLE 1. Export of vesicle-associated DNA by bacteria

Species and strain	Result of gram stain	Presence of vesicles	Types of DNAs[a]
Agrobacterium tumefaciens 15955	−	+	CP
Bacillus cereus 14579	+	+	None
Bacillus subtilis 6051	+	+	None
Bordetella pertussis 3779	−	+	LC
Borrelia burgdorferi Sh-2-82	−	+	LP, CP, LC
Escherichia coli 11775	−	+	LC
Haemophilus influenzae clinical[b]	−	+	CP
Haemophilus parainfluenzae 33392	−	+	CP
Moraxella osloensis 19976	−	+	CP, LC
Neisseria gonorrhoeae 31426	−	+	CP
Pseudomonas aeruginosa 10145	−	+	LC
Salmonella typhimurium 13311	−	+	LC
Serratia marcescens 13880	−	+	LC
Shigella dysenteriae 13313	−	+	CP, LC
Shigella flexneri 29903	−	+	CP, LC
Staphylococcus aureus 12600	+	−	ND
Streptococcus sanguis 10556	+	−	ND
Yersinia pestis EV76	−	+	CP, LC

[a] Abbreviations: CP, circular plasmid; LP, linear plasmid; LC, linear chromosomal; ND, not determined.
[b] Clinical isolate, Community Medical Center, Missoula, Mont.

Occasional vesicles were also observed on the surfaces of certain gram-positive bacteria such as *Bacillus subtilis* and *Bacillus cereus*; however, log-phase staphylococci and streptococci apparently lacked attached vesicles (Table 1). Since previous studies showed that few vesicles are produced by lag-phase bacteria and since stationary-phase cultures contain products of autolysis (5), we examined only log-phase organisms. Therefore, the possibility remains that staphylococcal or streptococcal vesicles occur at other growth stages. When produced by the bacterial cultures, the vesicles were washed from cell surfaces and successfully concentrated by differential and sucrose density gradient centrifugation (Fig. 1b).

For each culture, the extracellular vesicles were polymorphic. When entire colonies were mounted and examined, cells with and without attached vesicles were often observed in the same preparations (data not shown), suggesting that vesicle formation by colonial bacteria is not synchronous. The percentages and spatial distributions of vesicle-forming bacteria within colonies were not determined.

Both agarose gel electrophoresis and electron microscopy were used to characterize DNAs associated with extracellular vesicles (4, 7). Vesicles were purified from vigorously growing cultures to minimize contamination of preparations by products of autolysis (4, 5). Furthermore, to destroy any extraneous DNA in the preparations and prevent possible capture of external nucleic acids by membrane vesicles into transformasomelike structures, the vesicles were exhaustively digested with DNase I before nucleic acids were extracted. Any DNA recovered from vesicles after this treatment was then compared by agarose gel electrophoresis with DNA from whole cells. Figure 2 shows a representative gel containing 2 μg of whole-cell DNA and the total of approximately 5 μg of DNA present in vesicle extracts from 10 confluent plates of *Agrobacterium tumefaciens* 15955.

All vesicle extracts from gram-negative bacteria had detectable DNA, while no extracellular nuclease-protected DNA was recovered from gram-positive bacteria (Table 1). Vesicle extracts typically contained either a full or a partial complement of parental-cell plasmid bands. Heterogenous

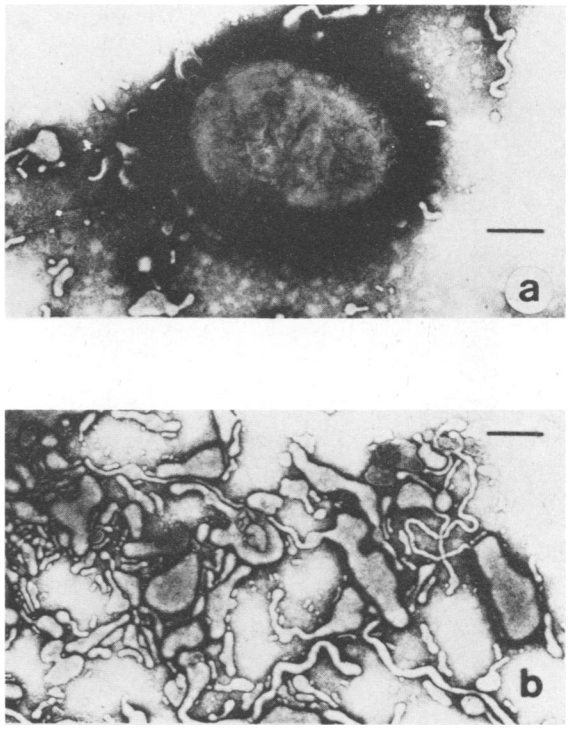

FIG. 1. Electron microscopy of cells and vesicles from log-phase cultures of *B. pertussis*. Bacterial colonies were bottled (a) or purified extracellular vesicles were adsorbed (b) onto Parlodion-coated grids, negatively stained with 0.5% ammonium molybdate, and observed by electron microscopy. Vesicles were commonly observed on and about gram-negative bacteria. Cell-free preparations containing numerous morphologically diverse vesicles were recovered from buffer-washed cells by differential and sucrose density gradient centrifugation (see text). Bars, 250 nm.

DNA fragments, presumably chromosomal, were observed in some vesicle preparations. No externally added plasmid DNA was detected when pBR322 was added to vesicle preparations prior to nuclease treatment and phenol extraction (data not shown).

The electrophoretic results were confirmed by the Kleinschmidt technique for nucleic acid electron microscopy (Fig. 2). The figure shows molecules recovered from excised agarose gel bands. Under the aqueous conditions used, double-stranded DNA is detectable as threadlike strands, while single-stranded DNA and RNA molecules collapse into characteristic aggregates (6). Duplex DNA was observed in all electrophoretically positive extracts. As reported previously (4, 7), supercoiled molecules were extracted from nuclease-treated vesicles, indicating that such plasmids were protected and did not receive even a single nick during treatment (6, 7).

These results showed that the export of DNAs within nuclease-resistant membrane vesicles occurs in a variety of gram-negative bacteria. The export process appears unique to gram-negative bacteria, since vesicle-associated DNA was exported by all species tested, whereas no nuclease-protected DNAs were recovered from the gram-positive bacteria tested. Such results are consistent with observations that protected gonococcal DNAs are enclosed within outer membrane vesicles, whereas extracellular cytoplasmic-membrane-derived vesicles fail to protect associated DNAs from exogenous nuclease (4).

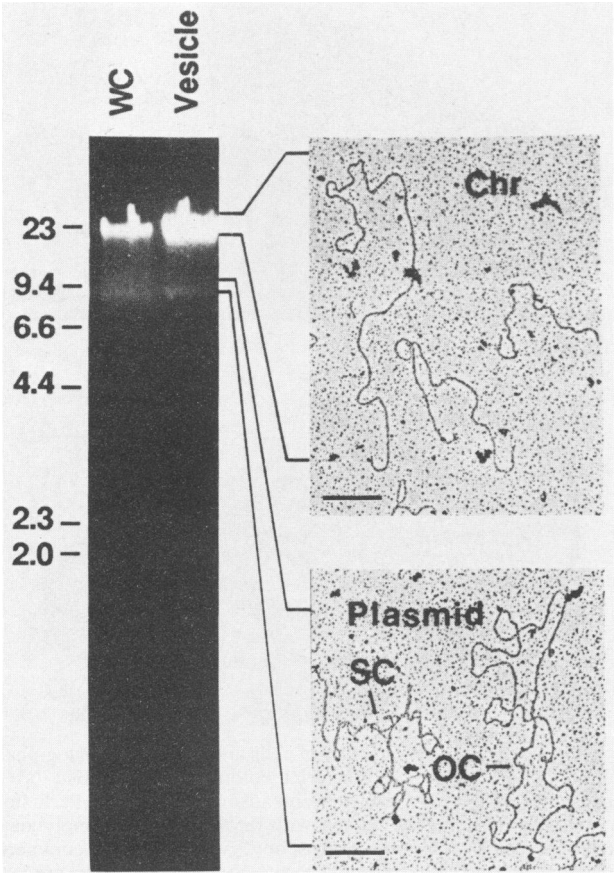

FIG. 2. Electrophoresis and electron microscopy of DNAs extracted from extracellular vesicles. DNA was extracted from cells and nuclease-treated vesicles of *A. tumefaciens* and characterized as described in the text (4). Heterogenous chromosomal fragments (Chr) and a plasmid band with a migration equivalent to linear fragments of 8.5-kilobase pairs were detected in whole-cell (WC) lanes and in vesicle extracts. The relative intensity of the whole-cell and vesicle bands suggested that heterogenous linear fragments were comparatively enhanced in vesicle extracts. Supercoiled (SC) molecules were resolved in preparations made from the excised vesicle plasmid band, indicating that the membrane-associated plasmids were protected from damage during exhaustive nuclease treatment. The nicked, open-circle (OC) molecules measured 3.7 μm in length, corresponding to approximately 11.1 kilobase pairs. Bars, 250 nm.

This study suggests that the export of vesicle-associated DNA is widespread among and may be characteristic of gram-negative bacteria. Possible functions of such export include genetic exchange between compatible organisms (4). Whether such exchange is limited to procaryotes or extends to transfections of eucaryotes, such as plants by *Agrobacterium* spp. (12) and yeasts by *Escherichia coli* (8), is unclear. Given the present data showing vesicle-mediated DNA export by several gram-negative pathogens, the possible effects of the DNA on infected hosts need consideration.

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